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RAC3 down-regulation sensitizes human chronic myeloid leukemia cells to TRAIL-induced apoptosis

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Abstract **The nuclear receptor coactivator RAC3 plays important roles in many biological processes and tumorigenesis. We found that RAC3 is over-expressed in human chronic myeloid leukemia cells K562, which are normally resistant to TRAIL-induced apoptosis. RAC3 down-regulation by siRNA rendered these cells sensitive to TRAIL-induced cell death. In addition to the up-regulation of TRAIL receptors, the process involves Bid, caspases and PARP activation, loss of mitochondrial membrane potential, and release of AIF, cytochrome c and Smac/DIABLO to the cytoplasm. We conclude that RAC3 is required for TRAIL resistance and that this anti-apoptotic function is independent of its role in hormone receptor signaling.**

Keywords: Nuclear receptors coactivator; RAC3; Apoptosis; Leukemia; TRAIL; NF-κB

1. Introduction

The steroid receptor coactivator SRC/p160 family (SRC-1/NCoA1; SRC-2/TIF2/GRIP-1; SRC-3/p/CIP/RAC3/ACTR/AIB-1/TRAM-1) interacts with nuclear receptors (NRs) and enhance their transactivation in a ligand-depend manner [1,2]. Two members of SRC family, SRC-1 and receptor associated coactivator-3 (RAC3), are also known to contain an intrinsic histone acetyltransferase activity [3,4] and all p160 proteins can recruit other general coactivators with enzymatic activity like CBP/p300, p/CAF, CARM-1 and PRMT1 [5], required for chromatin remodelling. It has been suggested that RAC3 could be more promiscuous than other p160 coactivators enhancing the transcription of non-NR proteins including NF-κB [6] and CREB [7]. Moreover, we have previously shown that RAC3 could be found associated with a protein complex containing the estrogen receptor in addition to NF-κB, that could regulate the expression of genes involved in cell proliferation [8]. RAC3 gene is highly overexpressed in several tumors, including breast and ovary tumors [9], endometrium [10], gastric [11], hepatocellular carcinoma [12], and prostate [13]. Its activity has been linked to proliferative processes [14,15].

FasL, TNF-α and TRAIL [tumor necrosis factor (TNF)-related apoptosis-inducing ligand] are ligands of a family of receptors with death domains. The binding of these molecules to their specific receptors causes, in responsive cells, the receptor oligomerization and triggers the activation of the cytoplasmic caspase machinery, resulting in disruption of normal cellular and nuclear morphology followed by DNA fragmentation [16]. TRAIL binds to five receptors: four of which are membrane-bound and one soluble receptor [17]. Two of these receptors, death receptor 4 (DR4) and -5 (DR5) are agonistic receptors containing a cytoplasmic death domain activated by TRAIL. Upon binding of TRAIL homotrimer to DR4 or DR5, which induces trimerization of the receptors, a death-inducing signaling complex (DISC) is formed. The adaptor protein FADD is then recruited to the complex, facilitating incorporation of the initiator procaspase-8 prodomain into the DISC. This leads to activation of procaspase-8, a potent activator of downstream effector caspases e.g., caspase-3, -6, and -7 [17]. TRAIL is a potent inducer of tumor-specific apoptosis in vitro and in vivo, which appears to induce apoptosis preferentially in tumor cells versus normal cells. The resistance of cells to TRAIL seems to be tightly regulated and dependent of the cell-type and the cell-differentiation [18].

Because resistance to apoptosis causes the failure in leukemia treatment, as in other tumor therapies, there is important to find the molecular mechanisms responsible of such response, in order to enhance the sensitivity to pro-apoptotic signals. In this work, we provide evidence that human chronic myeloid leukemia (CML) K562 cells express high levels of the p160 coactivator RAC3 as compared with positive control human breast tumor T-47D cells [19]. Moreover, RAC3 down-regulation by siRNA sensitized K562 cells to activation of the extrinsic/receptor-mediated cascade by TRAIL by inducing a potent cell death signal through a mechanism involving enhanced expression of DR4/DR5, and activation of caspase cascades and mitochondrial damage.

Therefore, our findings suggest that RAC3 is an interesting molecule to be analyzed in several leukemic cells as a possible anti-apoptotic mediator.

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Abbreviations: RAC3, receptor associated coactivator-3; TRAIL, tumor necrosis factor (TNF)- related apoptosis-inducing ligand; FLIP, FLICE inhibitory protein; AIF, apoptosis-inducing factor

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2. Materials and methods

2.1. Cells and cell culture

K562 cells were obtained from American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 supplemented with sodium pyruvate, MEM essential vitamins, L-glutamate, penicillin, streptomycin, and 10% fetal bovine serum (Gibco, Invitrogen, Carlsbad, CA). Human embryonic kidney HEK293 cells were grown in Dulbecco's modified Eagle's medium High Glucose (DMEM) (Gibco Laboratories, Grand Island, NY) supplemented with 10% FCS, penicillin (100 U/ml) and streptomycin (100 mg/ml). The human breast tumor T47D cells were grown in Dulbecco's modified Eagle's F12 medium (DMEM-F12) (Gibco Laboratories) supplemented with 10% FCS, penicillin (100 U/ml) and streptomycin (100 mg/ml). All the cells were maintained in a 37 °C, 5% CO_2, fully humidified incubator. The pSilencer-RAC3 small interfering RNA (siRNA) plasmid was prepared using Ambion system following the manufacturer's protocol according to the previously reported sequence [14] and a control not specific siRNA was obtained from Dharmacon Research Inc.. K562 cells stably over-expressing RAC3-siRNA and their control counterparts were selected by sub-culturing the cells on selection medium containing G418 antibiotic (Invitrogen).

2.2. Drugs and Chemicals

TRAIL/Apo2L was purchased from Alexis (Carlsbad, CA, USA) and stored in aliquots at −80 °C. Unless stated, reagents were obtained from Sigma–Aldrich (St. Louis, MO), BD-Pharmingen (San Diego, CA) or New England Nuclear (Boston, MA).

2.3. Assessment of apoptosis

Apoptotic cells were evaluated by Annexin V/propidium iodide (BD PharMingen) and 7-AAD staining, according to the manufacturer's instructions as previously described [20]. In these studies, $1–2 \times 10^5$ cells were harvested for each experimental condition. Analysis was carried out using a Becton Dickinson FACScan cytofluorometer (Mansfield, MA, USA). Morphologic assessment was evaluated by Wright-Giemsa–stained cytospin preparations.

2.4. Assessment of mitochondrial membrane potential ($\Delta\psi_m$)

At the indicated intervals, cells were harvested and 2×10^5 cells were incubated with 40 nmol/l of the lipophilic fluorochrome $DiOC_6$ (15 min, 37 °C). Loss of mitochondrial membrane potential was determined by flow cytometry as previously described [21].

2.5. Analysis of cytosolic cytochrome c, Smac/DIABLO and apoptosis-inducing factor (AIF)

A previously described technique was used to isolate the S-100 (cytosolic) cell fraction of treated cells [21]. For each condition, 30 μg of protein isolated from the S-100 cell fraction were separated and detected by Western blot as described.

2.6. Western blot analysis

Whole-cell pellets were washed and resuspended in PBS, and lysed with loading buffer (Invitrogen) as previously described [20]. Thirty micrograms of total protein for each condition were separated by 4–12% bis-Tris NuPAge precast gel system (Invitrogen) and electroblotted to nitrocellulose. After incubation with the corresponding primary and secondary antibodies, blots were developed by enhanced chemiluminescence (New England Nuclear).

2.7. Antibodies for Western blot analysis

Primary antibodies for the following proteins were used at the designated dilutions: RAC3, TIF2 and SRC-1 (1:4000; Santa Cruz); poly- (ADP)ribose polymerase (1:1000; BioMol, Plymouth Meeting, PA); pro-caspase-3, cytochrome *c* and pro-caspase-9 (1:1000, BD-PharMingen); procaspase-8 (1:2000; Alexia Corporations, San Diego, CA); Bid (1:1000; Cell Signaling, Beverly, MA); tubulin (1:4000; Calbiochem, San Diego, CA); AIF and FLIP, (1:1000; Santa Cruz); Secondary antibodies conjugated to horseradish peroxidase were obtained from Kirkegaard and Perry Laboratories Inc. (Gaithersburg, MD).

2.8. Surface expression analysis of DR4 and DR5

Cells (1×10^6) were washed two times with PBS and then incubated with DR4 or DR5 specific antibodies conjugated with FITC (Alexis) in PBS for 30 min at room temperature. After washing for three times, cells were analyzed on FACScan flow cytometer. Samples with an unspecific mouse IgG1 antibody were used as a control.

2.9. Measurement of NF-κB activity

Nuclear protein was extracted using Nuclear Extract Kit (Active Motif) and NF-κB activity was determined by using an enzyme linked immunosorbent assay (ELISA) Kit TransAM NF-κB p65 Transcription Factor Assay Kit (Active Motif), according to manufacturer's instructions. Briefly, the activated form of NF-κB that is present in nuclear extracts was detected by using an anti-p65 specific antibody that recognizes the NF-κB bound to a consensus DNA oligonucleotide immobilized in a 96-well plate. Addition of a secondary antibody conjugated to horseradish peroxidase provides sensitive colorimetric readout by spectrophotometry.

2.10. Statistical analysis

The significance of differences between experimental conditions was determined using ANOVA and Tukey–Kramer Multiple Comparisons Test for unpaired observations.

3. Results

3.1. RAC3 is over-expressed in K562 cells

RAC3 is over-expressed in several hormone-dependent and –independent tumors [9–13]. To determine the protein levels of p160 coactivator RAC3 in K562 cells, Western blot analysis was performed comparing levels of this protein in human HEK293 cell line which have almost undetectable nuclear receptor coactivator levels (unpublished data), a positive control of RAC3 over-expression human breast cancer T47D cells [19] and K562 cells. As shown in Fig. 1A, K562 cells displayed higher levels of RAC3 than in normal HEK293 cells but similar to the positive control T47D cells. In view of these observations and to investigate the role that RAC3 may play in K562 cells sensitivity to a pro-apoptotic stimulus, stable K562 clones expressing low levels of coactivator were generated. K562 cells were transfected with a vector carrying either a control small interfering RNA (K562/con) or a RAC3 small interfering RNA (siRNA) and two clones with different RAC3 expression levels, i.e. a moderate expressing clone (#10) and a very low expressing clone (#4) were selected (Fig. 1B). Importantly, RAC3 down-regulation did not affect the protein levels of the two other p160 coactivators, SRC-1 and TIF2 as previously described for this specific sequence of siRNA [14] (Fig. 1B).

3.2. Loss of resistance to TRAIL-induced cell death by RAC3 down-regulation

To investigate the effect of RAC3 down-regulation in TRAIL-induced cell death, RAC3 siRNA clones and the wild-type K562 cells were exposed to different concentrations of TRAIL including 10, 25, 75, 100 and 250 ng/ml. We observed that starting with TRAIL 25 ng/ml, RAC3 down regulation sensitized K562 cells to TRAIL-induced apoptosis (Fig. 2A). For example, after 16 h treatment with TRAIL 25 ng/ml, while no differences were observed in the percentage of apoptosis in K562/con (2.8%) or the wild-type cells (2.9%), a marked increase was determined in both RAC3 siRNA clones (i.e., 67.8% and 57.4%, low and medium RAC3 clones, respectively; Fig. 2A). A time-course analysis of TRAIL-induced lethality showed a rapid and signif-

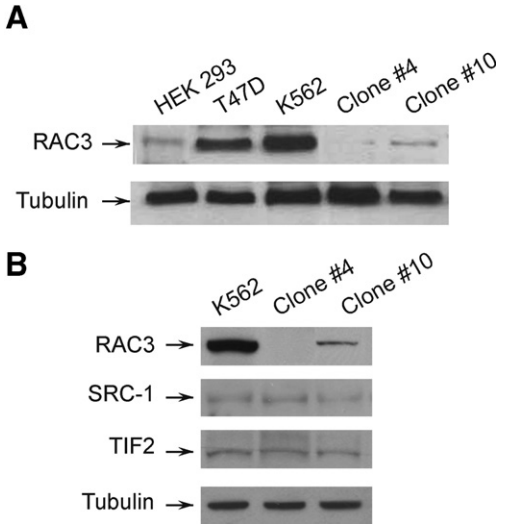

Fig. 1. RAC3 is over-expressed in K562 human leukemia cells and its down-regulation by siRNA does not modify p160 coactivators SRC-1 and TIF2 protein levels. (A) Western blot analysis of RAC3 in HEK293, T47D, K562/wild-type and stably K562 RAC3-siRNA transfected clones #4 and #10. (B) Western blot analysis of basal p160 coactivators RAC3, SRC-1 and TIF2 in K562 cells stably transfected with a RAC3-siRNA (clone #4 and #10) and the wild-type cells. The results of a representative study are shown; two additional experiments yielded similar results.

icant increase in cell death starting after 4 h of TRAIL addition (Fig. 2B). Interestingly, differences in apoptosis appeared dependent on RAC3 expression levels, i.e. higher TRAIL-induced lethality correlated with lower RAC3 levels, clearly indicating that the presence or absence of RAC3 plays a critical role in TRAIL-induced cell death.

3.3. RAC3 down-regulation inhibits NF-κB basal activity and increases the sensitivity to TRAIL-induced activation

In previous studies we have demonstrated that RAC3 is a NF-κB coactivator [6]. The anti-apoptotic role of this transcription factor is well-known [22]. In order to determine whether the NF-κB activity was affected by RAC3 down-regulation, the levels of active nuclear NF-κB were analyzed by ELISA. In these experiments, K562 wild-type, K562/con and RAC3-siRNA #4 and #10 clones were stimulated during 1 h with 25 ng/ml of TRAIL and then, the nuclear extracts were analyzed. It is important to notice that K562 wild-type cells are known to display high constitutive levels of NF-κB activity associated with the expression of Bcr-Abl [23]. As shown in Fig. 3, the basal NF-κB activity appeared significantly inhibited in RAC3-siRNA clones compared to K562/con and K562/wt cells. Moreover, NF-κB activity was directly related to the expression levels of RAC3 as significant differences were evident between medium and low expression in RAC3-siRNA clones. Interestingly, upon TRAIL stimulation, RAC3-siRNA clones displayed increased NF-κB activity to values comparable to those of K562/con and K562/wt cells, which themselves did not show significant changes.

3.4. TRAIL-induced apoptosis in low level RAC3 cells involves activation of both caspases and Bid and PARP cleavage

Western blot analysis of lysates obtained from RAC3-siRNA, K562/con and K562 wild-type cells exposed to 25 ng/ml

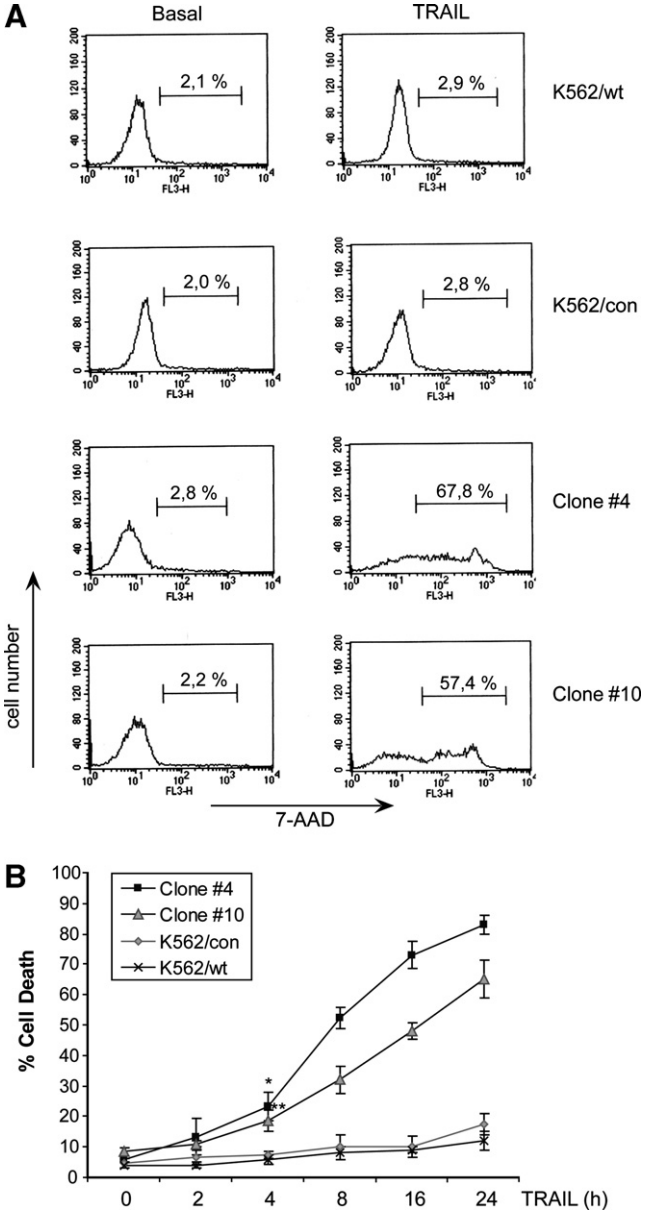

Fig. 2. RAC3 down-regulation sensitivizes K562 cells to TRAIL-induced apoptosis. (A) RAC3-siRNA clones #4 and #10, K562/wt and K562/con cells were exposed to 25 ng/ml TRAIL for 16 h after which cells were stained by 7-AAD and monitored for cell death by flow cytometry. (B) Time-course analysis of cell death induced by 25 ng/ml TRAIL as determined by flow cytometry analysis in RAC3-siRNA clone #4, clone #10, K562/wt and K562/con cells. Cells were collected at the indicated time-intervals (0–24 h) and stained with annexin V/PI. Data shown represent the means ± S.D. from three independent experiments. *, ** Significantly higher than values determined in K562 after 4 h exposure; $P < 0.01$ and <0.05, respectively.

of TRAIL during 8 and 16 h revealed that enhanced TRAIL-mediated cell death in RAC3-siRNA #4 and #10 clones was accompanied by cleavage/activation of procaspase 8 and the caspase-8 substrate Bid as compared with K562/con and K562/wt cells, where no changes were detected (Fig. 4). In addition, activation of caspases-9 and -3 as well as degradation of caspase-3 substrate poly ADP-ribose polymerase (PARP) were clearly observed in both RAC3-siRNA clones; moreover, the level of caspases and Bid activation were

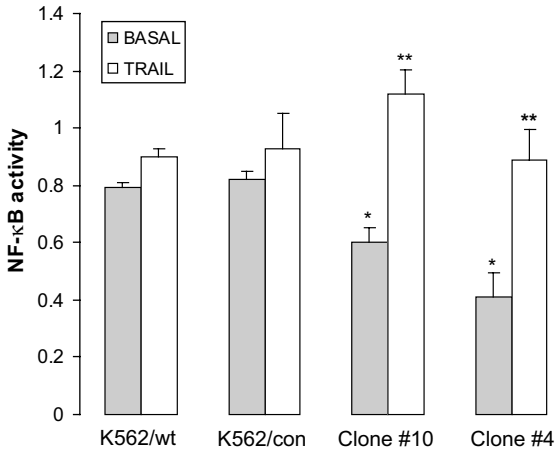

Fig. 3. Modulation of NF-κB activity by RAC3 down-regulation and TRAIL stimulation. K562/wt, K562/con and RAC3-siRNA cells were treated with 25 ng/ml TRAIL for 1 h. Then, cells were collected, nuclear extracts prepared and NF-κB activity was assessed by an ELISA kit. Data shown represent the means ± S.D. from three independent experiments. *Significantly less than basal condition in K562 cells, $P < 0.01$; **significantly higher than the corresponding clone basal condition, $P < 0.01$.

correlated with RAC3 levels and apoptosis described before. Thus, these results are consistent with TRAIL mediating activation of the receptor apoptotic cascade, a process involving also caspases-9 and -3 which are related to the intrinsic, mitochondrial apoptotic pathway.

3.5. TRAIL-induced apoptosis of cells with low levels of RAC3 involves the activation of the mitochondrial pathway

It is well established that TRAIL can induce cell death by activating mitochondrial-dependent and – independent pathways [24]. Therefore, and taking into account the results described above (Fig. 4), we analyzed whether the intrinsic/mitochondrial pathway was also activated by TRAIL as part of the mechanism of induction of cell death. The mitochondrial membrane potential ($\Delta\psi_m$) was determined in RAC3-siRNA clones and their counterpart K562 wild-type and K562/con cells after stimulation with TRAIL for 2, 4, 8, 16 and 24 h. Loss of $\Delta\psi_m$ was evaluated by flow cytometry after staining with the lipophilic fluorochrome $DiOC_6$ dye. As shown in Fig. 5A, exposure of RAC3-siRNA clones resulted in a clear increase in loss of $\Delta\psi_m$ in RAC3-siRNA clones as compared to K562/wt and K562/con cells. Again, significant differences were observed in $\Delta\psi_m$ depending the level of expression of RAC3 (Fig. 5A). Other markers of mitochondrial integrity were then analyzed. Western blot analysis of cytosolic fraction (S100) revealed a significant increase in the amount of mitochondrial proteins AIF, cytochrome *c* and Smac/DIABLO released from the mitochondria to the cytosol (Fig. 5B); these proteins were detected in the cytosol early after addition of TRAIL (i.e., 4 h). Taking together these results indicate that RAC3 down-regulation sensitizes K562 cells to TRAIL-

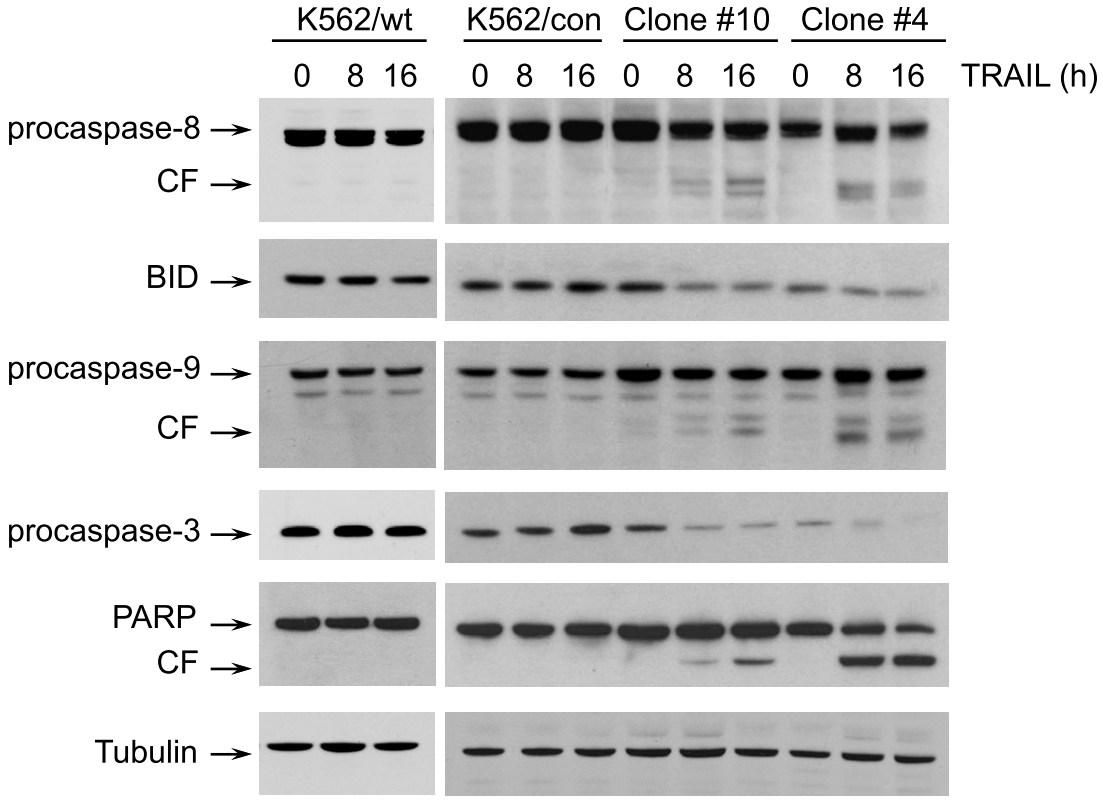

Fig. 4. TRAIL-induced activation of both caspases and Bid and PARP cleavage in RAC3-siRNA clones. K562/wt, K562/con cells and RAC3-siRNA stable clones #4 and #10 were exposed for 8 and 16 h to 25 ng/ml TRAIL. The antibodies for caspases recognize the cleaved fragment (CF) in addition to the un-cleaved pro-caspases. The results of a representative study are shown; two additional experiments yielded similar results.

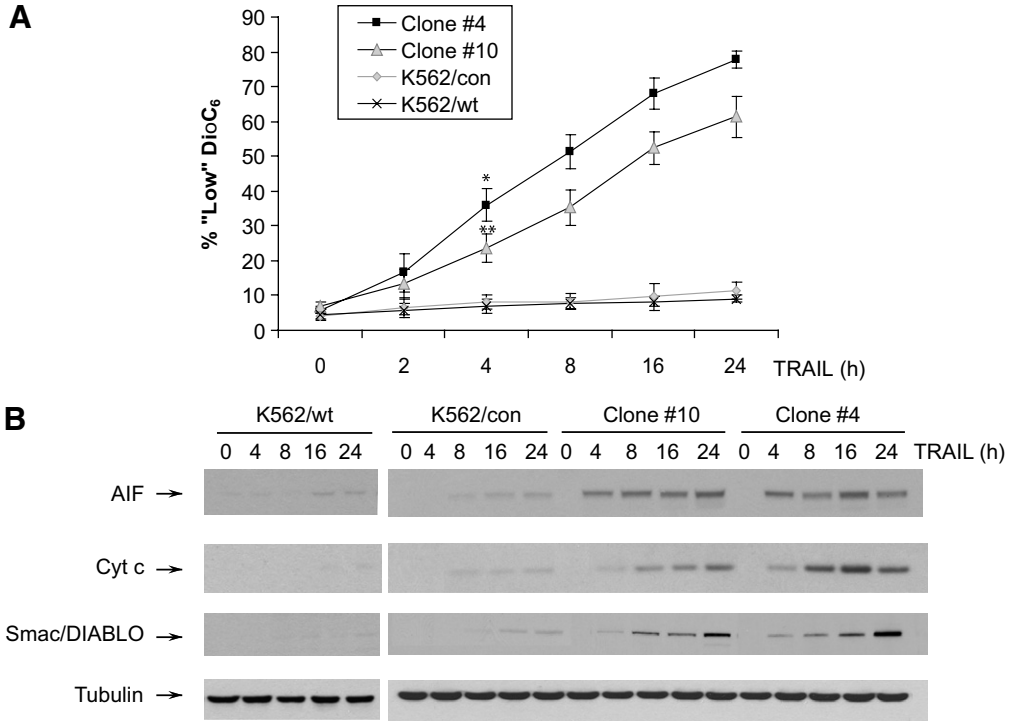

Fig. 5. Exposure to TRAIL induces mitochondrial damage in RAC3-siRNA clones. (A) K562 wild-type, K562/con cells and RAC3-siRNA clones #4 and #10 were treated with 25 ng/ml TRAIL for 2, 4, 8, 16 and 24 h after which cells were incubated with $DioC_6$ and loss of $\Delta\psi_m$ was determined by flow cytometry. Data shown represent the means ± S.D. from three independent experiments. *, **Significantly higher than values determined in K562 after 4 h exposure; $P < 0.001$ and <0.01, respectively. (B) K562/con cells and RAC3-siRNA stable clones #4 and #10 were exposed for the indicated time-intervals to 25 ng/ml TRAIL after which proteins were extracted from the cytosolic S-100 fraction, separated by SDS–PAGE gels and probe with the corresponding antibodies. The results of a representative study are shown; two additional experiments yielded similar results.

induced apoptosis trough a mechanism involving activation of both membrane and mitochondrial pathways.

3.6. *RAC3 down-regulation diminishes cFLIP$_L$ expression and increases the expression of DR4 and DR5 TRAIL receptors*

Recent studies have shown that sensitization of human colon cancer cells to TRAIL-mediated apoptosis involves a diminished expression of FLICE inhibitory protein (FLIP) [25], a protein that interferes with DISC activation and is a potent inhibitor of the TRAIL-mediated receptor pathway [26]. Moreover, the expression of cFLIP is transcriptionally regulated by NF-κB [27,28]. Attempts were then undertaken to determine whether modulation of cFLIP$_L$ may be involved in TRAIL-induced lethality in RAC3-siRNA cells. As shown in Fig. 6A, RAC3-siRNA clones showed a dramatic, RAC3-dependent reduction in cFLIP$_L$ expression compared to K562/con cells.

It has been also shown that increased sensitivity to TRAIL-induced cell death in various cell types, including leukemia cells, may stem from increased expression of TRAIL receptors [29,30]. Therefore, the levels of membrane-associated death receptors 4 (DR4) and -5 (DR5) were determined by flow cytometry. As shown in Fig. 6B, RAC3-siRNA clone #10 cells displayed a notorious increase (shift to the right) in the amount of both DR4 (left panel) and DR5 (right panel) associated to the cell membrane compared to their counterparts K562 wild-type and K562/con cells. Similar results were observed in RAC3-siRNA clone #4 cells (data not shown).

Together, these results suggest that RAC3, by controlling the basal NF-κB activity, the amount of membrane-associated DR4 and DR5 and by reducing the levels of the caspase-8 negative regulator cFLIP$_L$ may be playing a key role in modulating the sensitivity to TRAIL-induced apoptosis in an extrinsic/receptor-mediated signaling that involves the mitochondrial apoptotic pathway.

4. Discussion

Failure to achieve complete and durable responses from cancer chemotherapies is a common clinical problem that limits the curative potential of anti-cancer drugs in clinical oncology. The human chronic myeloid leukemia K562 cells are resistant to several pro-apoptotic drugs effect that has been attributed, amongst others, to a high expression of the multidrug transporter P-glycoprotein, responsible of the multidrug resistance (MDR) [31]. In this regard, several chemotherapeutic drugs are not effective and the development of new specific strategies to attack cancer cells is required. Such is the case of apoptosis induced by TRAIL. In the present study we have found that human CML K562 cells over-express the p160 family coactivator RAC3 with levels similar to those observed in mammary tumor cells [9]. Although not all tumor cells are sensitive to TRAIL-induced apoptosis and the mechanism underlying TRAIL-resistance is unclear, we determined that once the levels of RAC3 are down-regulated, K562 cells, which are

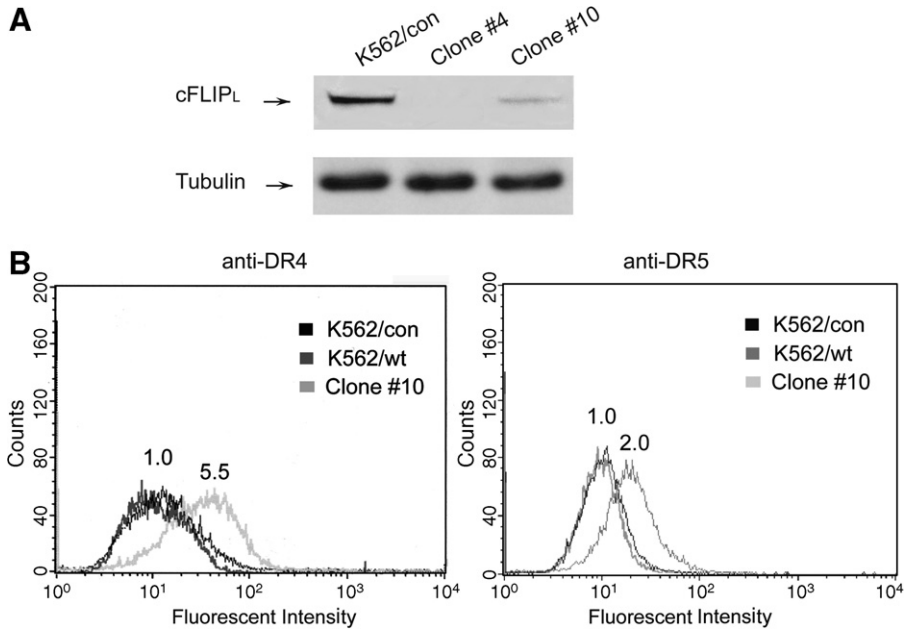

Fig. 6. RAC3-downregulation diminished $cFLIP_L$ expression and increased surface expression of DR4 and DR5. (A) Levels of $cFLIP_L$ were determined by Western blot in K562/con cells and RAC3-siRNA clones. (B) Analysis of surface TRAIL receptors DR4 and DR5 by flow cytometry in K562/wt, K562/con cells and RAC3-siRNA clone #10. Cells were incubated with anti-DR4 and -DR5 specific antibodies as described in Section 2. The relative fluorescence intensity (RFI) is indicated for each condition. The results of a representative study are shown; three additional experiments yielded similar results.

naturally resistant to TRAIL-induced apoptosis, became sensitive. Consistent with previously described mechanisms of TRAIL-induced cell death [32], both the membrane and mitochondrial pathways appeared rapidly activated upon TRAIL exposure in K562 cells with RAC3 down-regulation. Our results indicate that down-regulated RAC3-mediated increased sensitivity to TRAIL reflects changes at different levels including TRAIL-receptors, which leads to formation of more DISC, and, importantly, to the release of one of the most critical negative regulators, $cFLIP_L$. Thereby, our results also suggest that lack of sensitivity shown by K562 cells to TRAIL may be strictly correlated to RAC3 over-expression which in turn, as the present findings indicate, plays a critical role in maintaining high basal levels of NF-κB. Although we did not analyzed other leukemic cell lines, these observation contributes to the knowledge of possible molecular mechanisms that may be involved in other cell lines, including leukemia models, that are resistant to several drugs or specific ligand bound receptors. Accordingly, the levels of RAC3 in other MDR cells deserve to be investigated.

It has been previously demonstrated that NF-κB inhibits TNF-α-induced cell death [33]; moreover, constitutive activation of this transcription factor also prevents TRAIL-induced apoptosis in renal cancer cells, effect that is reverted by inhibiting NF-κB which restored the sensitivity to TRAIL [34]. In this work, we described for the first time that K562 cells express high levels of the RAC3 coactivator, similar to those observed in human breast tumor T-47D cells, that constitute a positive control of coactivator over-expression [19]. In addition, and accordingly to our previous findings, RAC3 functions as a NF-κB coactivator [6]. Based in these observations, it is plausible to expect high and/or constitutive NF-κB activity in these cells. However, whether this is a direct effect associated to high levels of RAC3 or it reflects regulatory mechanisms driven by the activity of Bcr-Abl present in these cells, remains to be determined.

The present findings indicate that RAC3 down-regulation affected the basal NF-κB activity, since when we analyzed NF-κB activity in response to TRAIL, only RAC3 siRNA clones showed clear activation of NF-κB to values similar to those present in either K562 wild-type or K562/con cells. These data may be interpreted either as a consequence of low levels of TRAIL receptors, which may lead to very modest pro-apoptotic, and consequently pro-NF-κB effects of TRAIL, or to high constitutive NF-κB activity related to the presence of Bcr-Abl. Nonetheless, what our results indicate is that under RAC3 down-regulation, TRAIL-induced NF-κB activity becomes clear and may be explained as the result of lower basal activity together with higher levels of TRAIL receptors that enhance the sensitivity to this signal. Finally, the present results show that RAC3 may represent an important cancer resistance mediator and has the potential of being explored in leukemia cells as a possible target for cancer therapies.

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